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Influence of yeast strains on managing wine acidity using *Lactobacillus plantarum*

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A B S T R A C T

Lactobacillus plantarum has been used for deacidifying wines through malolactic fermentation (MLF), and more recently for acidifying them. The species *Oenococcus oeni* is mainly responsible for MLF in wines at a pH below 3.5. However, the *Lactobacillus* and *Pediococcus* species can carry out this reaction in wines at a higher pH. MLF generally begins once yeasts have completed alcoholic fermentation, but nowadays some winemakers prefer carrying out MLF simultaneously to alcoholic fermentation. The advantages of this strategy are shorter times in which to complete wine vinification and better wine color stabilization. *Lactobacillus plantarum* is preferred to *O. oeni* for performing early MLF because it catabolizes hexoses homofermentatively, which prevents acetic acid from increasing. In recent years, wine composition has changed as a result of climate change. High temperatures during the maturation time of grapes give rise to musts with low contents of organic acids, a high pH and high sugar contents, which give wines with low acidity, a high ethanol content and a faded color. Although several physical or chemical procedures can increase acidity, it can also be achieved by using microorganisms capable of transforming sugars into acids (biological acidification); e.g. lactic acid bacteria (LAB). The advantages of this procedure are increased wine acidity and reduced ethanol content. When early MLF or biological acidification is desired, yeasts and bacteria must develop together in grape must. In this work, we studied the consequences of the interactions of different combinations of two strains of *L. plantarum* and six commercial strains of Saccharomyces cerevisiae during white grape must fermentation. We demonstrated that managing wine acidity is possible by co-inoculating *L. plantarum* and *S. cerevisiae*. MLF was performed with any LAB/yeast combination, but the degree of biological acidification depended on the combination. LAB growth was more negatively affected by yeast than vice versa. The best combination to manage wine acidity was *L. plantarum* E4608 and Uvaferm VRB®.

1. Introduction

In recent years, wines composition has been modified as a result of climate change. High temperatures during grape ripening results in low acidity and high sugar content musts, which lead to high pH, high ethanol, faded color and no freshness in wines. High pH wines are highly susceptible to microbiological spoilage, which can cause organoleptic depreciation, and even the accumulation of toxic products, such as ethyl carbamate and biogenic amines (Mira de Orduña, 2010). Several physical, chemical and biological methods have been used to increase wine acidity. Chemical methods are the most widely used and are based on the addition of authorized organic acids, such as malic acid, tartaric acid, citric acid or lactic acid. Physical methods, like electrodialysis and ion exchange resins, can also be used. All these practices are strictly regulated by legislation (OIV, 2015). The addition of yeasts or bacteria to produce organic acids is another biological method that has been used by several authors to increase wine acidity (Kapsopoulou, Mourtzini, Anthoulas, & Nerantzis, 2007; Lucio, 2014; Mora, Barbas, & Mulet, 1990; Onetto & Bordeu, 2015; Val, Ferrer, & Pardo, 2002). The advantage of this method is that it is not subjected to any regulation and can be used to produce organic wines. Lucio (2014) and Onetto and Bordeu (2015) increased wine acidity by inoculating strains of *Lactobacillus plantarum,* prior to *S. cerevisiae,* in grape must.

In the vast majority of red, and in some white, winemaking processes, the elimination of malic acid is sought because this acid confers wine a sour herbaceous flavor (Chidi, Rossouw, Buica, & Bauer, 2015).

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Malic acid is a substrate that can be used for lactic acid bacteria (LAB), which causes instability of bottled wine. For all these reasons, the presence of this acid is not desired, even though the objective is to acidify wine. Generally, the *O. oeni* species is responsible for malolactic fermentation (MLF) in wines with a pH below 3.5. MLF begins once yeasts complete alcoholic fermentation (AF). For a few years, some winemakers prefer performing MLF before or simultaneously to AF. In this case, *L. plantarum* is preferred to *O. oeni* because it catabolizes hexoses homofermentatively, which prevents acetic acid from increasing (Lerm, Engelbrecht, & du Toit, 2010; Onetto & Bordeu, 2015). In addition, wines in which simultaneous AF/MLF have been performed are fruitier, less buttery and more complex and structured that those in which these processes sequentially occur (Lerm, Engelbrecht, & du Toit, 2010).

When bacteria and yeast coexist during grape must fermentation, interactions are established that can be beneficial or detrimental to both or to only one kind of microorganisms (Boidron, 1969; Fornachon, 1968; Gilis, Delia-Dupuy, & Strehaiano, 1996). The nature of LAB-yeast interactions is highly dependent on the involved strains (Álvarez-Martín, Flórez, Hernández-Barranco, & Mayo, 2008; Dicks, Todorov, & Endo, 2009; Lerm et al., 2010; Nehme, Mathieu, & Taillandier, 2008). Therefore, when early MLF or biological acidification (BA) is desired, the choice of a compatible couple of strains is a key factor to ensure that these processes take place without compromising AF.

Antagonism between yeasts and LAB during winemaking is a well-studied phenomenon. Currently during AF, the yeast population is around 10^8 UFC/mL, whereas the LAB concentration is around 10^2-10^3 UFC/mL (Lonvaud-Funel, 1999). The reasons to explain these facts are competence for nutrients and the synthesis of toxic compounds by yeasts (Dicks et al., 2009; Fugelsang & Edwards, 2007; Lerm et al., 2010; Nehme, Mathieu, & Taillandier, 2010). In fermenting grape must, yeasts assimilate nutrients (some are essential growth factors for LAB) faster than LAB given their bigger size and mass (Beelman, Keen, Banner, & King, 1982). A higher metabolic flux of sugars in *S. cerevisiae* than in bacteria results in the yeast population overcoming the bacteria population (Dashko, Zhou, Compagno, & Piškur, 2014). In addition, yeasts can synthesize inhibitory metabolites for LAB (Boidron, 1969; Fornachon, 1968; Gilis et al., 1996; Lafon-Lafourcade, 1973; Lemaresquier, 1987; Mayer, 1978; Wibowo, Fleet, Lee, & Eschenbruch, 1988). Between the inhibitors produced by yeast are ethanol, SO_2, medium chain fatty acids, β-phenylethanol, succinic acid and certain peptides (Capucho & San Romao, 1994; Caridi & Corte, 1997; Comitini, Ferretti, Clementi, Mannazzu, & Ciani, 2005; Guerzoni, Sinigaglia, Gardini, Ferruzzi, & Torriani, 1995; Lafon-Lafourcade, Geneix, & Ribéreau-Gayon, 1984; Osborne & Edwards, 2007; Suzzi, Romano, & Zambonelli, 1985).

However, yeasts can also stimulate LAB growth. During AF, yeasts can release nutrients, such as vitamins and amino acids, which promotes bacterial growth (Divies, Feuillat, Cavin, Guilloux-Benatier, & Guzzo, 2003). This phenomenon is accentuated by the lysis of yeasts at the end of AF. At this time, vitamins, nitrogen compounds (amino-acids) polysaccharides (glucans) and cell wall proteins (mannoproteins) are released (Charpentier & Feuillat, 1993; Fornairon-Bonnefond, Camarasa, Moutounet, & Salmon, 2001; Llaubères, Richard, Lonvaud, Dubourdieu, & Fournet, 1990). Moreover, glucans and mannoproteins can adsorb medium chain fatty acids, which detoxify the medium by stimulating LAB growth in this way. Some of these polymers can also be additional nutrient sources for LAB if they are hydrolyzed by a specific yeast or LAB enzymes (Guilloux-Benatier et al., 2003; Guilloux-Benatier, Son, Bouhier, & Feuillat, 1993).

LAB can also inhibit yeast. Although some authors believe that LAB do not affect the growth of yeasts during AF, others have demonstrated that LAB accelerate the death rate of yeast at the end of AF (King & Beelman, 1986). At this point, the negative interactions of LAB on yeasts could be due to either the high consumption rate of nutrients by LAB, which results in a deprived situation for yeasts, or the synthesis of inhibitory products for yeasts, such as acetate or propionate (Chowdhurry, Watier, Leguerinel, & Hornez, 1997). These reasons have been reported as the causing agents of stuck fermentations (King & Beelman, 1986). The majority of studies into the LAB/yeasts interaction have been performed with sourdough microbiota or wine *O. oeni* and *S. cerevisiae* strains. However, very little information has been published on wine *L. plantarum*/*S. cerevisiae* strains.

The aim of this work was to study the influence of six commercial *S. cerevisiae* strains on the growth and acidity management of two *L. plantarum* strains. To accomplish this objective, the growth kinetics of yeasts and bacteria, the quantification of lactic, citric and malic acids, and pH values were determined at the end of the experiments. In addition, residual sugars, ethanol and glycerol were quantified to know the effect of the presence of LAB on yeast metabolism.

2. Material and methods

2.1. Microbial strains and culture conditions

The employed bacterial strains were *Lactobacillus plantarum* E4607 and E4608, which came from a selection program directed to find proper *Lactobacillus* strains capable of performing BA in low acidity wines (Lucio, 2014; Lucio, Pardo, Krieger-Weber, Heras, & Ferrer, 2016). LAB strains were routinely grown in MRS broth (Scharlau) supplemented with 0.5 g/L of L-cysteine (Merck) and cultivated without shaking at 28 °C.

Six commercial *Saccharomyces cerevisiae* yeast strains were used to perform AF: Uvaferm VN®, Uvaferm VRB®, Lalvin K1™, Enoferm M2™, Lalvin 4600® and Lalvin QA23™ (Lallemand). Yeast strains were routinely grown in YPD broth (20 g/L of glucose, 20 g/L of mycological peptone, and 10 g/L of yeast extract) under shaking conditions at 28 °C. Yeasts were inoculated in grape must according to the manufacturer's indications.

2.2. Laboratory microvinifications

Laboratory microvinifications were performed in Airén white grape must (original grape must was 4-fold concentrated and then diluted in distilled water before use) supplemented with 0.3 g/L of Nutrient VitEnd® (Lallemand). The pH of the must was adjusted to 3.5 and it was sterilized at 115 °C for 30 min. Grape must composition was 94.52 g/L of glucose, 113.23 g/L of fructose, 0.72 g/L of malic acid and 0.34 g/L of citric acid.

Each L. *plantarum* E4607 and E4608 strain was inoculated in 750 mL of grape must at a cell density of 2×10^6 colony-forming units (CFU)/mL, and was allowed to grow for 3 days at 28 °C. Volumes of 50 mL of these LAB cultures were distributed into seven flasks. Six of these flasks were inoculated separately with six commercial yeast strains at a cell density of 2×10^6 CFU/mL, whereas the other were left without yeast and was used as a control to monitor LAB growth in the absence of yeasts. Six other flasks containing 50 mL of grape must were inoculated only with yeast, one yeast per flask, to monitor yeast growth in the absence of LAB. One additional grape must flask with neither LAB nor yeasts was incubated under the same conditions as the other control to check if grape must sterility continued throughout the experimental time. All the flasks were incubated for 15 days at 28 °C. Experiments were done in duplicate.

2.3. LAB and yeast growth kinetics monitoring

The growth of the inoculated LAB and yeast during microvinifications was determined by the plating method. Daily samples of inoculated grape musts were taken, diluted and spread on MRS plates (Scharlau), to which 0.5 g/L of L-cysteine (Merck) and 0.15 g/L of Actistab (Gist-Brocades) were added for the LAB counts, and onto YPD agar for the yeast counts. Plates were incubated at 28 °C for 3–6 days.

2.4. Chemical characteristics of the obtained wines

The samples obtained at the end of assays were filtered through 0.22-μm filters and analyzed by High Performance Liquid Chromatography in an Agilent 1200 system, equipped with a high performance automatic injector, a refractive index detector, a variable wavelength detector G1314B/G1314C, an isocratic pump, and a thermostatic compartment (Agilent 1200). Five microliters of the filtered samples were injected using a precolumn AMINEX HPX-87H (BIORAD) bound to two ion exclusion columns of 30 nm × 7.8 nm AMINEX HPX-87-H (BIORAD). The running time was 40 min. Samples were eluted with 0.75 mL/L of 85%phosphoric acid (Panreac) at 70 °C. The flow rate was 0.7 mL/minute. The glucose, fructose, ethanol, glycerol, malic acid, lactic acid and citric acid concentrations were quantified by this method.

The pH values of the final wines were measured by an electrode Sentix81 (WTW) coupled to a pH-meter pH330i (WTW).

2.5. Statistical analysis

A one-way ANOVA analysis of the physico-chemical data of the resulting wines was performed. Three analyses (each per assayed condition: without LAB, with *L. plantarum* E4607 and with *L. plantarum* E4608) were performed to know if the yeasts gave rise to significant differences in the final characteristics of wines. The confidence limit was set at 95%.

3. Results

3.1. Growth kinetics of yeast and bacteria

The populations of *L. plantarum* strains E4607 and E4608 increased 1.5 logarithmic units before yeast inoculation. Strain E4607 reached the maximum biomass before E4608, although the maximum viable cell concentration was similar for both strains (Fig. 1). The growth of the LAB strains was severely limited after yeast inoculations. The viable cell concentrations of both the LAB strains sharply dropped while yeasts performed AF. At the end, only very low concentrations of E4608 viable cells (lower than 1×10^3 CFU/mL) were recovered in some experiments. The viable LAB concentrations detected 4 days after yeast inoculation ranged between 2×10^4 and 1×10^1 CFU/mL for strain E4607 (Fig. 1A), and between 5×10^5, and 9×10^3 CFU/mL for strain E4608 (Fig. 1B), depending on the inoculated yeast strain, which meant that the percentage of mortality was 99.93% or higher. The yeast strains showing a lower inhibitory effect to E4607 were Lalvin QA23™ and Enoferm M2™, whereas the most inhibitory one was Lalvin K1™. Uvaferm VN® and Uvaferm VRB® were the strains with the lowest inhibitory effect on E4608. Lalvin K1™ was the yeast strain that promoted a higher mortality rate in both strains LAB E4607 (99.99% of mortality) and E4608 (99.98% of mortality).

Although yeast growth was almost unaffected by the LAB strains on the first 4 days after co-inoculation, higher death rates were observed in the presence of bacteria after this time (Fig. 2). The degree of yeast mortality induced by the LAB strains depended on the yeast strain. Both the LAB strains inhibited mainly Lalvin K1™ (Fig. 2C), Enoferm M2™ (Fig. 2D) and Lalvin QA23™ (Fig. 2F). The growth curves of Uvaferm VRB® (Fig. 2B) and Lalvin 4600® (Fig. 2E) were similar in the absence or presence of the LAB strains. The microbial combination that assured optimal yeast and LAB growth was E4608 and Uvaferm VRB®.

3.2. Influence of co-inoculation on acid concentration, pH, ethanol and glycerol

The chemical composition of the co-inoculated wines was not that different, regardless of the yeast/LAB combinations. However, large differences in the sugars, acids, ethanol and glycerol concentrations were found between the media inoculated only with a LAB or a yeast strain (Fig. 3). The ANOVA analysis results are depicted as letters on each type of bar in Fig. 3A–F. Different types of letters are used for the three treatments: without LAB, with E4607 and with E4608 bacteria. Different letters on the same type of bar indicate significant differences at the 95% probability.

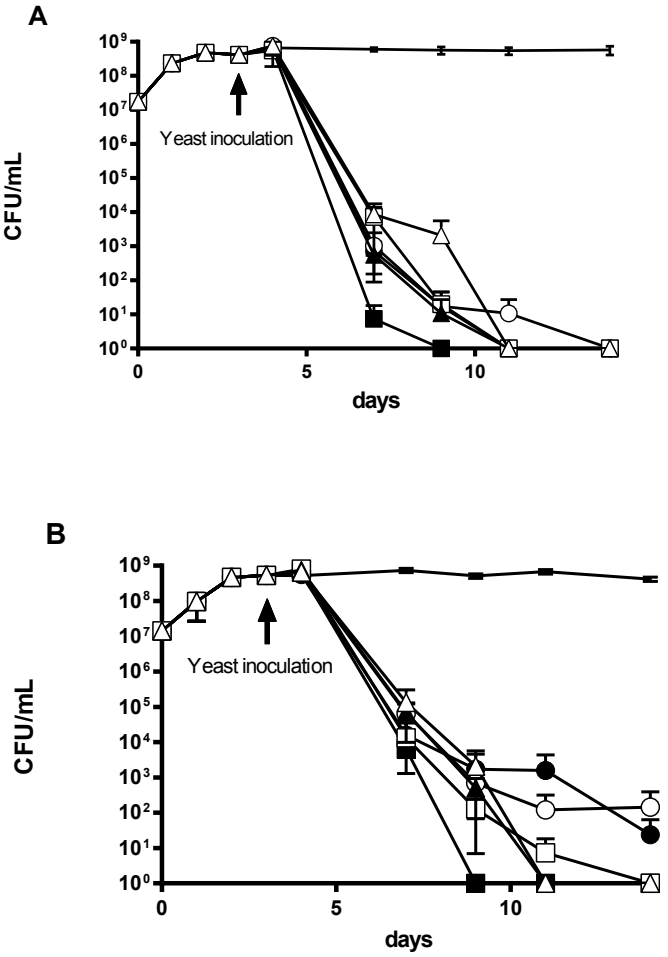

Fig. 1. LAB growth in co-culture with different commercial yeast strains and under the pure-culture condition (A) *L. plantarum* E4607, (B) *L. plantarum* E4608. (-) without yeasts, (●) with Uvaferm VN®, (○) with Uvaferm VRB®, (■) with Lalvin K1™, (□) with Enoferm M2™, (▲) with Lalvin 4600®, (△) with Lalvin QA23™.

3.2.1. Metabolism of sugars and acids by yeasts in pure-culture vinifications

The residual sugar concentrations of the wines inoculated only with yeasts ranged between 1 and 1.6 g/L (Fig. 3A). The driest wine was that fermented with Enoferm M2™, whereas the medium one with a higher residual sugar concentration was that fermented with Lalvin 4600®. Significant differences in the ethanol concentrations of the final wines were recorded, and the highest concentration was found in that fermented with Enoferm M2™, and the lowest one in that fermented with Lalvin K1™ (Fig. 3B). The strains that produced more glycerol were Enoferm M2™, and Lalvin 4600®, whereas the least producing ones were Lalvin K1™ and Uvaferm VN® (Fig. 3C). The wines fermented with Enoferm M2™ contained slightly more ethanol and slightly less glycerol than that fermented with Lalvin 4600®. No clear relationships between consumed sugars and ethanol and glycerol production were detected. Regarding the L-malic acid of the fermented wines, this acid remained constant along the AF in all cases, except in those fermented with Lalvin QA23™ and Enoferm M2™, in which yeasts partially degraded this acid (Fig. 3D). Small quantities of lactic acid were detected in the wines after

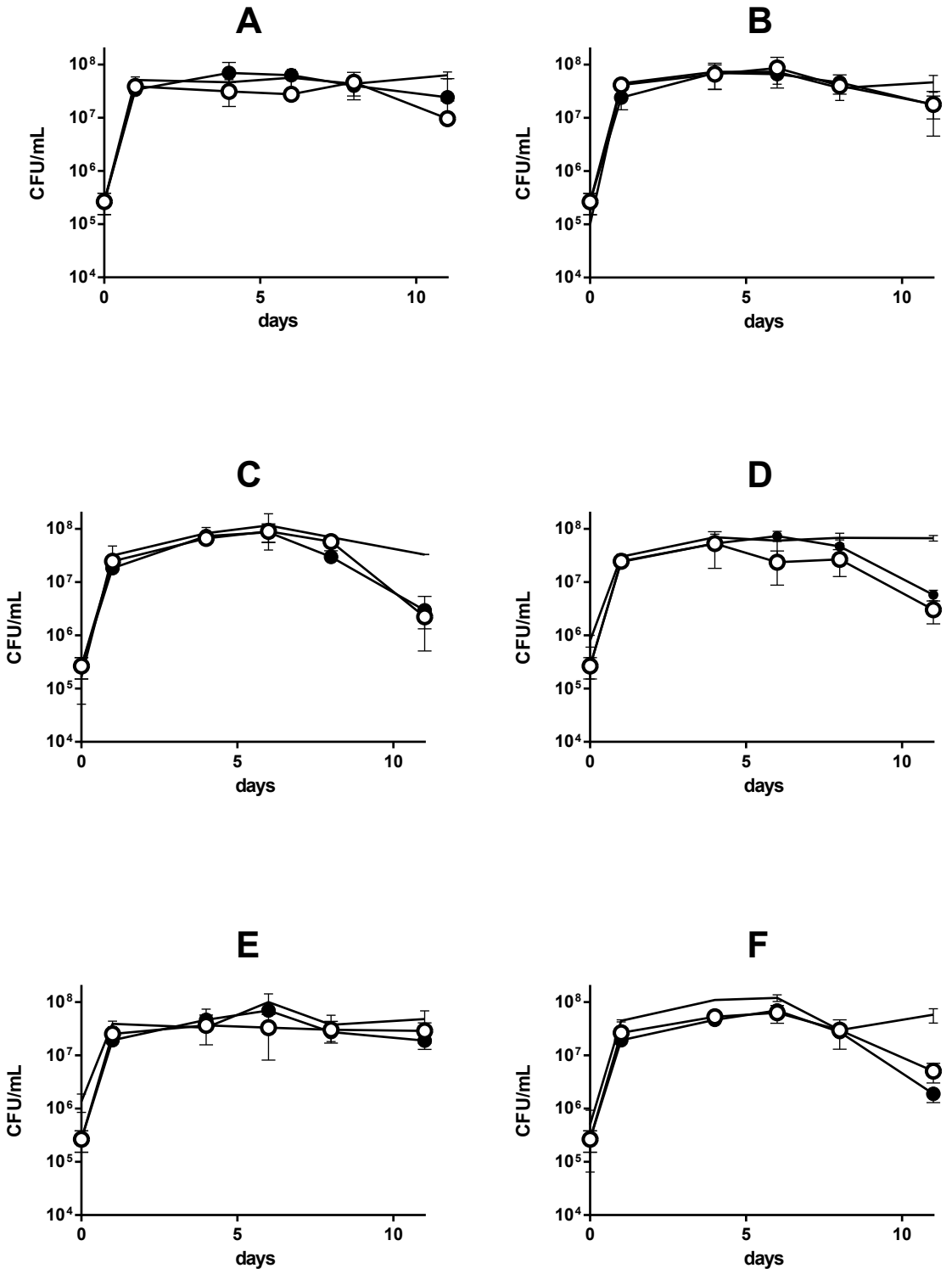

Fig. 2. Growth of commercial yeast strains populations in the co-culture with LAB and under the pure-culture condition (A) Uvaferm VN®, (B) Uvaferm VRB®, (C) Lalvin K1™, (D) Enoferm M2™, (E) Lalvin 4600®, (F) Lalvin QA23™. (-) without LAB, (●) with *L. plantarum* E4607, (○) with *L. plantarum* E4608. Day zero in this figure corresponds to the day of yeast inoculation, 3 days later than the bacteria inoculation.

AF. The wine with the highest concentration of this acid was that fermented with Lalvin QA23™ (1.3 g/L) (Fig. 3E). Yeasts were able to produce citric acid at concentrations that ranged from 0.6 to 0.9 g/L, depending on the strain (Fig. 3F). The best producer was Enoferm M2™ and the lowest producer was Lalvin QA23™.

3.2.2. Metabolism of sugars and acids by LAB in pure-culture vinifications

The fermented grape musts in which only the LAB strains were inoculated showed high residual sugar concentrations, 178 g/L and 173 g/L for those fermented with *L. plantarum* E4607 and E4608, respectively. No ethanol, glycerol and malic acid were detected in any of the fermentations conducted only by LAB. However, lactic acid was found at concentrations ranging from 17.2 to 18.8 g/L in those

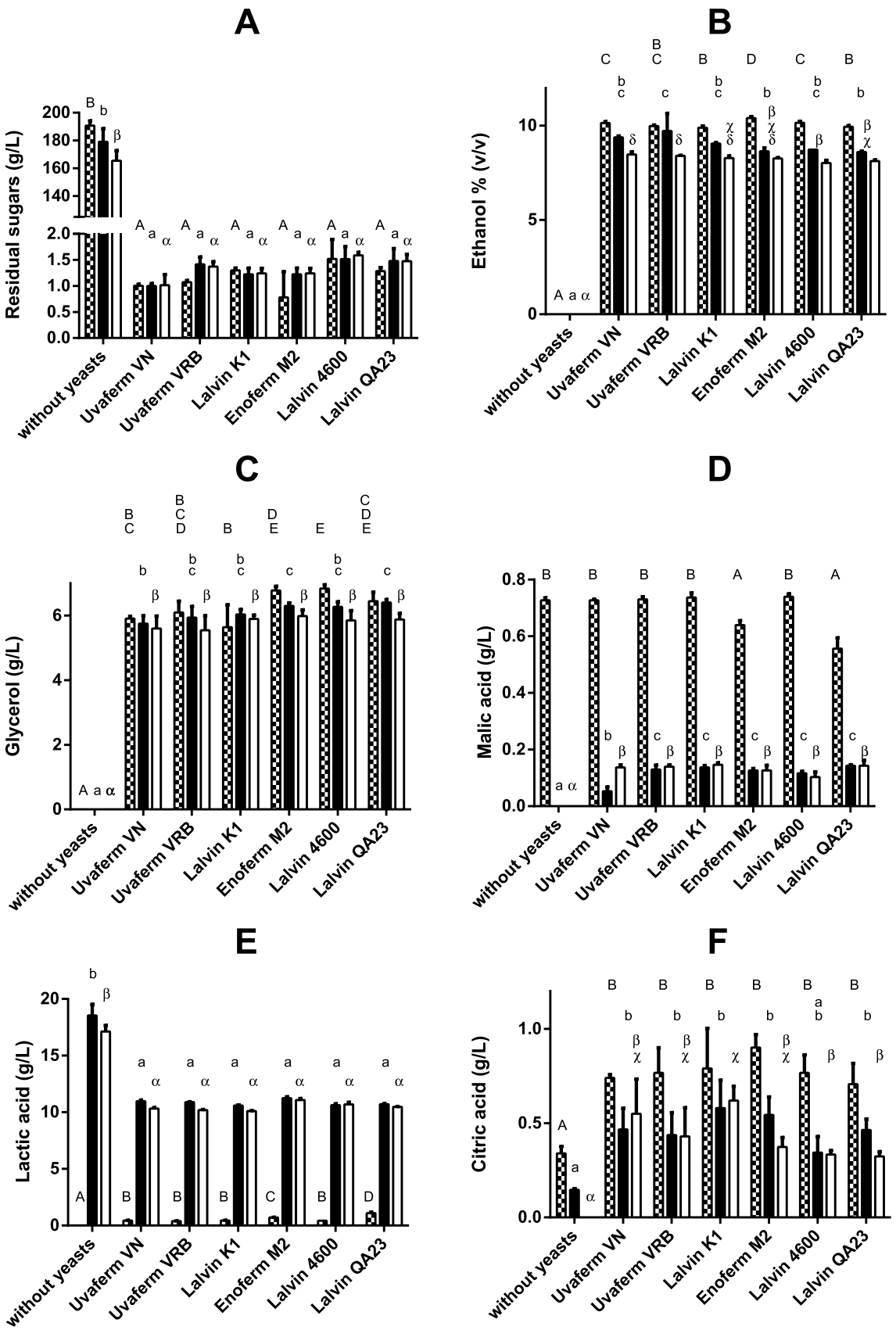

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Fig. 3. Chemical composition of the final wines obtained by microvinification using different combinations of yeast/LAB (A) residual sugars (g/L), (B) ethanol (% v/v), (C) glycerol (g/L), (D) malic acid (g/L), (E) lactic acid (g/L) and (F) citric acid (g/L). Different capital letters on the bars corresponding to the experiments "without LAB" express significant differences in the chemical composition of the wines fermented with distinct yeasts. Different lowercase letters on the bars corresponding to the experiments "with *L. plantarum* 4607" express significant differences in the chemical composition of the wines fermented with distinct yeast/*L. plantarum* E4607 couples. Different Greek letters on the bars corresponding to the experiments "with *L. plantarum* 4608" express significant differences in the chemical composition of the wines fermented with distinct yeast/*L. plantarum* E4608 couples.

inoculated with strains 4608 and 4607, respectively. Citric acid, originally present in the Airén white grape must (0.34 g/L), was totally consumed by *L. plantarum* E4607 and E4608.

3.2.3. Metabolism of sugars and acids by yeasts and LAB co-inoculated vinifications

The wines co-inoculated with LAB and yeasts showed a residual sugar concentration below 1.7 g/L. In general, these concentrations were similar between the vinifications performed with the different yeast/LAB couples (no significant differences were found), and very similar or slightly higher than those found in the yeast pure-culture vinifications. The ethanol and glycerol contents of the co-inoculated wines were lower than those inoculated only with yeast (between 0.25 and 1.75% lower). The wines fermented with strain E4608 were less alcoholic and contained less glycerol than those inoculated with the other LAB strain. Whereas 100% L-malic acid was degraded in the wines inoculated only with LAB, 10% of this acid remained in the co-inoculated wines. The lactic acid concentration was more than 10-fold higher in the co-inoculated wines (10–12 g/L) than in the yeast pure-culture fermentations (0.5–1.3 g/L), but was lower than in the LAB pure-culture ones (17–18 g/L). No significant differences between yeast/LAB couples were observed. Citric acid concentrations were always lower in the co-inoculated wines (0.3–0.6 g/L) than in those fermented only with yeast (0.7–0.9 g/L), but were higher than those inoculated only with LAB (0–0.13 g/L).

The pH values of the co-inoculated wines were 0.2 units lower than the yeast pure-culture fermentation, and 0.18 units higher than those fermented only with bacteria. The LAB/yeast couples that gave the highest and lowest pH wines were E4607 and Uvaferm VN®, and E4608 and Lalvin 4600®, respectively (Fig. 4).

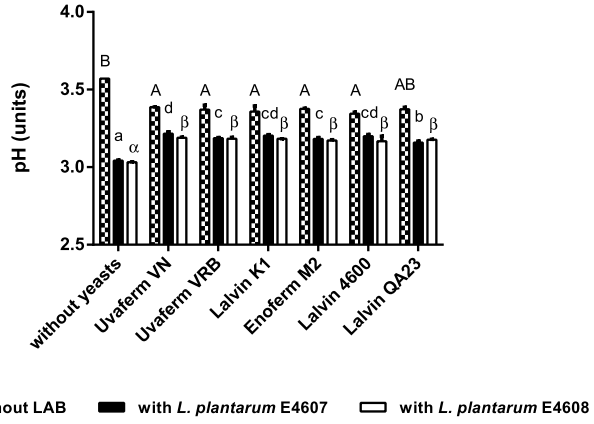

Fig. 4. pH values of the final wines obtained by microvinification using different yeast/LAB combinations. Different capital letters on the bars corresponding to the experiments "without LAB" express significant differences in the pH of the wines fermented with distinct yeasts. Different lowercase letters on the bars corresponding to the experiments "with *L. plantarum* 4607" express significant differences in the pH of the wines fermented with distinct yeast/*L. plantarum* E4607 couples. Different Greek letters on the bars corresponding to the experiments "with *L. plantarum* 4608" express significant differences in the pH of the wines fermented with distinct yeast/*L. plantarum* E4608 couples.

4. Discussion

Yeasts dramatically influenced LAB survival. Three days after yeast inoculation, mortality rates higher than 99% were recorded for both the *L. plantarum* strains, although the death rates of strain E4608 were lower than those of E4607. Differences in LAB mortality rates depended on the co-inoculated yeast strain. As all the yeasts grew similarly, the differences in the LAB mortality rates could not be attributed to distinct yeast population pressures on bacteria. Some authors have pointed that one of the reasons to explain a negative interaction of yeast on bacteria is the yeast's rapid uptake of essential amino acids, vitamins and growth factors for LAB from grape must (Lafon-Lafourcade, 1973; Lerm et al. (2010); Wibowo et al., 1988). Different uptake rates of essential growth factors by yeasts could result in various degrees of nutritional deficiency of fermenting grape must. However, lack of nutrients would probably affect the active growth phase more than the stationary growth phase of bacteria (Alexandre, Costello, Remize, Guzzo, & Guilloux-Benatier, 2004; Fugelsang & Edwards, 2007). Some authors have described other factors as being responsible for the death of bacteria: ethanol, medium chain fatty acids and SO_2 produced by yeast. The ability to produce ethanol is a general trait of *S. cerevisiae,* but ethanol yield and the capability to produce fatty acids or SO_2 are strain-dependent characters. The final ethanol concentration in wines was extremely similar, regardless of the strain used for fermentation, which means that it must not be mainly responsible for LAB death. In fact the more inhibitory yeast strain/s produced similar or lower ethanol concentrations than the less inhibitory one/s. Although the final ethanol concentration and different ethanol production rates may influence LAB viability. Capucho and San Romao (1994) have reported that 4% (v/v) ethanol inhibits the growth rate of bacteria. However, our experiments revealed that bacteria were already in the stationary growth phase when yeasts began to produce ethanol. Chu-Ky, Tourdot-Marechal, Marechal, and Guzzo (2005) have described how ethanol shock of 10%–14% v/v results in instantaneous membrane fluidization, followed by rigidification and a drop in *O. oeni* cell viability. Similar changes in membrane fluidity might explain the death of our *L. plantarum* strains.

The yeasts used in this work differ in SO_2 production and compatibility with malolactic bacteria. Whereas the majority of yeast strains produce low SO_2 and do not interfere with MLF, Lalvin K1™ produces more SO_2 and is less compatible with malolactic bacteria (http://www.lallemandwine.com/es/products/wine-yeasts-es) (Anonymous, 2017), in fact, this strain caused the higher mortality in both LAB strains.

Another cause of LAB mortality could be the medium chain fatty acids synthesized by yeasts during AF (Alexandre et al., 2004; Capucho & San Romao, 1994). Fatty acids inhibit the ATPase activity of LAB, and thereby reduce the ability of bacteria to maintain an intracellular pH and the transmembrane proton gradient, which is essential to transport metabolites across the cell membrane (Capucho & San Romao, 1994; Carreté, Vidal, Bordons, & Constantí, 2002). This could be another fact to explain the death of bacteria, but the fact that the death rates of the LAB grown with Lalvin K1™ were higher than those grown with Lalvin QA23™, and both are unable to produce medium chain fatty acid (Krieger-Weber, 2017; personal communication), do not support this theory. Many studies have reported the effect of yeasts on LAB in winemaking, especially *S. cerevisiae* on *O. oeni*, but much less information is available about the effect of this yeast on *L. plantarum*. In three of the cases in our work, yeast growth did not differ so much in the co-inoculate or pure-cultured fermentations. However in the other

three, the death phase accelerated when *L. plantarum* was inoculated prior to yeasts. Beelman et al. (1982) and Alexandre et al. (2004) have also found that the death phase rate of a *S. cerevisiae* strain is higher when inoculated simultaneously with *L. oenos* (syn. *O. oeni*) in a defined synthetic medium. They postulate that the production of L-ornithine from L-arginine by *O. oeni* causes the death of yeast cells. Bonomo et al. (2017) have reported that 0.5–1 g/L of this amino acid inhibits natural and wild yeast infection in wine. Some authors have pointed out another reason to explain the negative effect of LAB on *S. cerevisiae:* a lower pH and the higher titrable acidity of wines in which both microorganisms coexist during AF (Fugelsang & Edwards, 2007; Huang, Edwards, Peterson, & Haag, 1996). In our case, all the LAB/yeasts co-inoculated wines showed lower pH values than those inoculated only with yeasts, according to the results reported by Onetto and Bordeu (2015). The final pH of wines resulted from lactic acid production and from malic and citric acid consumption, while acid production exceeded the acid consumption in the wines inoculated with bacteria, which resulted in lower pH wines.

The yeasts that showed early death phase were Lalvin K1™, Enoferm M2™ and Lalvin QA23™. The wines fermented with these yeasts and *L. plantarum* strains showed a higher concentration of citric acid (data from this work), acetate, malate and succinate (Krieger-Weber, 2017; personal communication) than those fermented with other yeast strains. Ullah, Orij, Brul, and Smits (2012) have studied the antifungal activity of weak organic acids (sorbic, benzoic, propionic, and acetic) on *S. cerevisiae*, as well as the mechanisms that yeast used to counteract their effects. They have found that, although the lipophobicity of the acids correlates with the acidification rate of the cytosol, it is the cell's ability to restore the internal pH that is the determinant factor to surpass growth inhibition, and this ability is related to membrane ATPase Pma1p activity. They have reported that long-term acidification is the major cause of growth inhibition under acetic acid stress. Other authors have also reported the negative effect of high producing acetic acid *Lactobacillus* strains on *S. cerevisiae* (Edwards, Reynolds, Rodriguez, Semon, & Mills, 1999; Fugelsang & Edwards, 2007). The early death phase of Lalvin K1™, Enoferm M2™ and Lalvin QA23™ in the presence of LAB might be due to higher lactic acid production, which could have the same effect pointed out by Edwards et al. (1999) and Fugelsang and Edwards (2007) for acetic acid.

Apart from studying the growth interactions between LAB and yeasts, we were interested in knowing how the yeasts/bacteria interaction affected the management of wine acidity by LAB. The interaction between *L. plantarum* and *S. cerevisiae* has been considerably less studied than that between *O. oeni* and *S. cerevisiae*, possibly because using the first couple to perform MLF is less extended in winemaking. In our study, we noticed that MLF was poorly influenced by the presence of yeast: only 10% malic acid remained nondegraded in the co-inoculated wines. This result agrees with those reported by Capucho and San Romao (1994), Edwards, et al. (1999) and Onetto and Bordeu (2015). On the contrary, yeasts greatly influenced wine acidification by LAB. The early death of bacteria promoted by yeasts resulted in wines with lower lactic acid concentrations than the *L. plantarum* pure-culture fermentations; the same results have been recorded by Onetto and Bordeu (2015). In short, co-inoculation affects the biological acidification of must by LAB, but not MLF. Co-inoculation also gives rise to less alcoholic wines, which is logical because LAB consume part of the grape must sugar for their own growth and produce lactic acid as a result. Onetto and Bordeu (2015) have pointed that the prior inoculation of *L. plantarum* to *S. cerevisiae* in grape must can be used not only as a BA technique, but also as a potential way to reduce alcohol in wines. BA might be a smart strategy to solve the problems of high pH wines from warm-climate regions: lack of freshness, high ethanol content, high susceptibility to microbial spoilage, and a higher risk of containing biogenic amines (Lucio, 2014). Using this strategy implies the co-existence of yeasts and bacteria and, hence, it must be assured that the interaction between both microorganism types is neutral or positive. Some authors have described a positive and negative effect between *S. cerevisiae* and *O. oeni* (Alexandre et al., 2004; King & Beelman, 1986; Nehme et al., 2008), but very few have studied the interactions between *S. cerevisiae* end *L. plantarum,* and their studies have focused mainly on metabolic activities (Dicks et al., 2009; Edwards et al., 1999; Essia Ngang, Letourneau, Wolniewicz, & Villa, 1990; Onetto & Bordeu, 2015), but not growth consequences. In our assays, the five different commercial yeast strains severely compromised the survival of the two *L. plantarum* strains, but at different degrees depending on the couple. A similar negative influence of yeasts on bacterial growth has been reported by Huang et al. (1996) and Berbegal et al. (2016). Berbegal et al. (2016) found that the early death of bacteria slightly affected malic acid degradation, but greatly affected lactic acid production, which agrees with our results. This could be due to the low malic acid concentration, and that it was rapidly metabolized by the *L. plantarum* strain before yeast inoculation. A small part of lactic acid came from malic acid, but the main production was dependent on sugar catabolism. Lower lactic acid concentrations were found in co-inoculated wines than in pure LAB inoculated wines as a result of the early LAB death caused by yeasts.

The effect of the co-inoculation of the *L. plantarum* and *S. cerevisiae/bayanus* strains on the acidification of wines has been studied by Val et al. (2002) and more recently by Onetto and Bordeu (2015). Val et al. (2002) have reported increases in titrable acidity from 1 to 1.6 g/L (expressed as tartaric acid) when the strains of *L. plantarum* were co-inoculated with the *S. cerevisiae/bayanus* strains in pilot scale fermentations. Onetto and Bordeu (2015) have found that the time lapse between the *L. plantarum* and *S. cerevisiae* inoculations was related to lactic acid yields. When both microorganisms were inoculated at the same time, the lactic acid produced from sugar catabolism was very limited (0.58 g/L), whereas if yeast was inoculated 10 days after the bacteria, lactic acid yield was much more higher (6.35 g/L). In our case, we obtained a higher lactic acid concentration in all the co-inoculation experiments, which delayed yeast inoculation only by days 3, and not by 10 days as the aforementioned authors did.

To manage wine acidity, we must take into account that MLF is easily performed by *L. plantarum*, regardless of the yeast used to carry out alcoholic fermentation. However, the yields of different organic acids depend on the LAB/yeast couple. Other products that derive from yeast metabolism rely on the combinations of both types of microorganisms. Our results confirm that the interactions between yeasts and LAB are negative. This negative influence is exerted mainly on LAB by yeasts. The nature of this negative effect remains to be elucidated.

5. Conclusions

We have demonstrated that co-inoculation dramatically influences LAB survival, but only slightly affects yeast growth, and dramatically influences lactic acid production, and slightly affects malic acid degradation by LAB. The beginning and the intensity of the LAB death phase were determined by the LAB/yeast combination. The characteristics of the co-inoculated wines also differed from those inoculated solely with yeasts. The main differences were less ethanol, glycerol and citric acid, which are typical metabolic products from yeasts. The most compatible couple was E4608 and Uvaferm VRB®. LAB inoculation did not affect the sugar consumption by yeast and the residual sugar concentrations were similar in both the co-inoculated and in pure-culture wines. Managing wine acidity is enabled by co-inoculating *L. plantarum* and *S.* cerevisiae. The couple showing the least negative interaction was *L. plantarum* E4608 and Uvaferm VRB®.

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